Covalent modification of engineered cysteines in the nicotinic acetylcholine receptor agonist-binding domain inhibits receptor activation

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We constructed and characterized a series of nicotinic receptor mutants with a cysteine substituted for one of the amino acid residues in the α-subunit between positions 183 and 198. This region of the receptor is known to participate in agonist binding and channel activation. The goal of this 'cysteine scanning mutagenesis' is to introduce the reactivity of a free thiol group into functionally important protein domains; modification of the introduced cysteines can then be used to probe the structure and function of the targeted region. Mutants were examined by co-expression with the β-, γ- and δ-subunits in *Xenopus* oocytes using two-microelectrode voltage clamp recording. Twelve of fourteen mutants expressed receptors with properties comparable with the wild-type, including sensitivity to reduction by dithiothreitol (DTT). This indicates that introduction of an additional cysteine within this region of the receptor did not interfere with formation of the native disulphide between αCys-192 and αCys-193. Only one mutation, αY198C, caused dramatic changes in the EC_{50} for acetylcholine (ACh) and in the sensitivity to DTT. We then examined the effects of the thiol modification and found two mutants, αH186C and αV188C, that showed significant decreases in responsiveness to ACh after exposure to methylmethanethiosulphonate (MMTS). Dose–response measurements show that exposure of αH186C mutants to MMTS causes a shift in apparent agonist affinity without changing the peak response, and this is not reversible by DTT. In contrast, the MMTS-treated αV188C mutants show changes in both apparent affinity and peak response which are readily reversed by DTT. Together, our data show that these two nearby residues occupy markedly different environments relative to the contact points for ACh. They also demonstrate that cysteine-substitution mutagenesis can be successfully applied to protein domains that include functionally important disulphides.

INTRODUCTION

Identification and structural characterization of the agonist-binding domain of ligand-gated ion channels is a major focus of molecular pharmacology. A prominent model system for these studies is the vertebrate muscle nicotinic acetylcholine (ACh) receptor (AChR). This receptor is a pentamer comprised of four subunits in a stoicheiometry of $\alpha_2\beta\gamma\delta$; each receptor is known to contain two ACh-binding sites [1]. Studies using affinity-labelling techniques demonstrated agonist-protectable covalent modification of cysteines at positions 192 and 193, and tyrosines at positions 190 and 198 of the α-subunit [2–4]. Western blotting using the competitive antagonist α-bungarotoxin, together with studies employing synthetic peptides, has confirmed that the region of the α-subunit between amino acids 173 and 201 contains major determinants of the agonist-binding site [5]. Affinity labelling has also identified two other regions of the α-subunit where lower levels of agonist-protectable labelling suggested a proximity to the ACh-binding site, at Tyr-93 and Trp-149 [6,7]. On the basis of these data, Dennis and colleagues [3] proposed a three-loop model in which three discontinuous regions of the α-subunit each participate in formation of the ACh-binding pocket. Another proposed model, based on electron microscopy of AChR from *Torpedo*, also suggests that the agonist-binding sites are completely contained within the α-subunits [8]. However, no model that includes only α-subunits can easily account for the differences in binding characteristics of the two agonist-binding sites.

More recent work has demonstrated that differences between the two agonist-binding sites are probably due to participation of the γ- and δ-subunits. Studies of αγ and αδ assembly intermediates showed that differing antagonist affinities could be ascribed to the presence of γ- or δ-containing dimers [9]. This finding was extended by functional studies of γ-less and δ-less AChRs expressed *in vitro* [10], and by studies of γ–δ chimaeras [11]. In addition, Czajkowski and Karlin [12] used a heterobifunctional cross-linker to show that a negatively charged residue in the δ-subunit was within 0.1 nm (10 Å) of the cysteine pair at positions 192 and 193. Together, these studies have led to models of the ACh-binding sites which presume that agonist-binding sites lie at the α–γ and α–δ subunit interfaces [13].

The combination of site-directed mutagenesis and heterologous expression has confirmed the importance of residues implicated by biochemical techniques in the interaction of agonists with the receptor. Positions in the α-subunit at which substitution has been shown to alter functional responses include the tyrosines at positions 190, 198 and 93, and the tryptophan at position 149 [14–16]. Functional analysis of mutants has also been used to confirm the importance of residues in the γ- and δ-subunits in determining binding characteristics of ACh [17] and curare [13]. Although the combination of mutagenesis and heterologous expression has provided information on the participation of specific residues in the process of agonist binding and activation, it does not directly allow for detailed modelling of secondary or tertiary structure of the binding site, nor does it provide data on conformational changes accompanying binding

Abbreviations used: ACh, acetylcholine; AChR, nicotinic acetylcholine receptor; DTT, dithiothreitol; MMTS, methylmethanethiosulphonate. Introduced mutations are identified by the subunit, the single-letter code and numerical position of the native residue, and the substituted amino acid; for example, αH186C is a mutant of the α-subunit with the histidine at position 186 replaced with a cysteine.

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and subsequent channel activation. We therefore decided to examine the role of the α-subunit in the structural and functional features of the ACh-binding domain by an alternative strategy: cysteine-substitution mutagenesis. As applied in other systems, this method provides an opportunity to combine the powerful techniques of molecular biology with a variety of biochemical and spectroscopic approaches.

Substitution mutagenesis involves the introduction of cysteine residues in domains of suspected structural or functional significance; the engineered cysteines provide sites at which chemical modifying reagents, metal ions or cross-linkers can then be used as functional and structural probes. This method has been successfully employed in studies of the bacterial aspartate receptor [18], *lac* permease [19] and carbonic anhydrase II [20]. The unique chemical reactivity of free thiol groups provides a target for chemical modification, as shown by studies of the channel-forming domain of the AChR [21]. Free thiol groups can also be used to create sites for metal ion binding [22] or allow formation of intra- or inter-molecular disulphide bonds [23]. As a first step toward application of this technique to the AChR agonist-binding domain, we prepared and characterized a series of mutant α-subunits with a single cysteine substituted between positions 183 and 198.

EXPERIMENTAL

Mutagenesis and *in vitro* RNA preparation

The pSP64-based plasmids containing the cDNAs for the α-, β-, γ- and δ-subunits of the mouse muscle nicotinic AChR were provided by Dr. J. Boulter (Salk Institute, La Jolla, CA, U.S.A.). The α-subunit cDNA was subcloned into a pSP64 plasmid containing *Xenopus* globin non-coding sequence (designated pSP64T [24]). Mutations were introduced into an α-subunit cDNA subcloned into pBluescript (Stratagene, La Jolla, CA, U.S.A.) using oligonucleotide-directed mutagenesis [25]. Mutations were confirmed by DNA sequencing [26], and the 800 bp *Bsu*36I–*Bgl*II fragment of the cDNA containing the mutation was subcloned into the α-pSP64T plasmid. RNAs encoding wild-type and mutant receptor subunits were transcribed as described previously [14]. After synthesis, RNA was purified with the RNAid kit (Bio101, La Jolla, CA, U.S.A.). Individual subunit RNAs were mixed in a ratio of $2\alpha:1\beta:1\gamma:1\delta$, and injected into *Xenopus laevis* oocytes as described previously [14]. Injected oocytes were maintained in ND-96 (96 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM Hepes/NaOH, pH 7.6) at 17 °C.

Electrophysiology

Whole oocyte currents were recorded 1–3 days after injection by two-electrode voltage clamp using an OC-725B amplifier (Warner Instruments, Hamden, CT, U.S.A.). Oocytes were continuously perfused with a modified low-Ca^{2+} saline (96 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 0.25 mM EGTA, 5 mM Hepes/NaOH, pH 7.4; ND-96E) and clamped at −40 mV for all experiments. Solutions containing ACh and dithiothreitol (DTT) in ND-96E were prepared daily; solutions of methylmethanethiosulphonate (MMTS) were prepared immediately before use. Current responses to a 30–45 s application of ACh were recorded for a range of concentrations; between each application the oocyte was washed for 2–5 min with ND-96E. MMTS and DTT applications were also followed by a 2–5 min wash. Current responses were normalized to the maximal response for each oocyte, and the data were then pooled for analysis using commercially available software (Origin; Microcal).

Toxin binding

Surface expression of AChRs was measured by incubation of injected oocytes (10–20 for each mutation) for 90 min at 22 °C in ND-96 containing 0.1% BSA and 10 nM ^{125}I-α-bungarotoxin (200–500 c.p.m./fmol; Amersham, Arlington Heights, IL, U.S.A.). Oocytes were washed with five changes of ND-96, and counted on a Micromedic 4/200 γ counter. Non-specific binding was estimated from binding to non-injected oocytes.

RESULTS

Our first step in the development of the cysteine-substitution approach was to confirm that mutant α-subunits containing an additional cysteine in the major ligand-binding domain are still capable of proper assembly and function. To assess this, dose–response relationships were measured for both mutant and wild-type AChRs using two-electrode whole-oocyte recording. Figure 1 shows a normalized comparison of the EC_{50} values that were obtained using this method. Of the 14 cysteine substitutions examined, only αY190C failed to give measurable responses to ACh. Of the 13 mutants that expressed functional channels, four differed by less than 2-fold from the wild-type EC_{50} value. Five of the mutant receptors had EC_{50} values 3–4-fold lower, and three were 3–6-fold higher than the wild type. One mutant, αY198C, had an EC_{50} that was 140-fold higher than the wild type. We also tested each of the mutants for cell surface expression of ^{125}I-α-bungarotoxin binding: all mutants with measurable current responses expressed cell surface toxin binding at levels comparable with wild-type levels (3–10 fmol per oocyte, 48 h after injection; results not shown). No detectable receptor expression was seen for the αY190C mutant using either the functional assay or the binding assay. On the basis of both surface labelling and peak current measurements, all of the

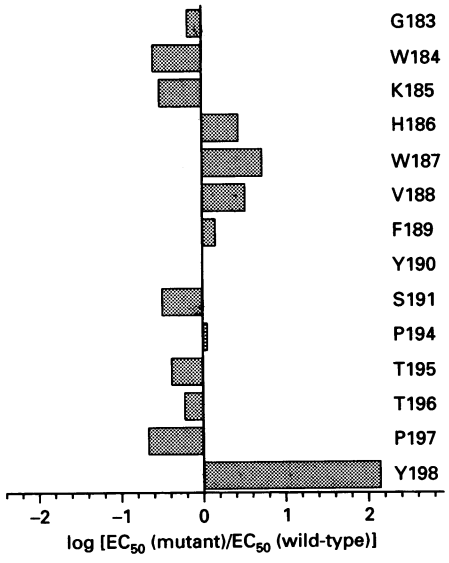

Figure 1 Effect of cysteine substitutions on the EC_{50} for ACh

Log ratios of mutant to wild-type EC_{50} values are plotted as a function of the position of the substituted cysteine in the α-subunit. Native residue and position are indicated on the right. EC_{50} values were obtained from logistic fits of normalized dose–response data pooled from at least three separate experiments. Errors are estimated from standard deviations of the calculated EC_{50} values. There was no detectable response with αY190C. αC192 and αC193, i.e. cysteines present in the wild-type receptor, are omitted.

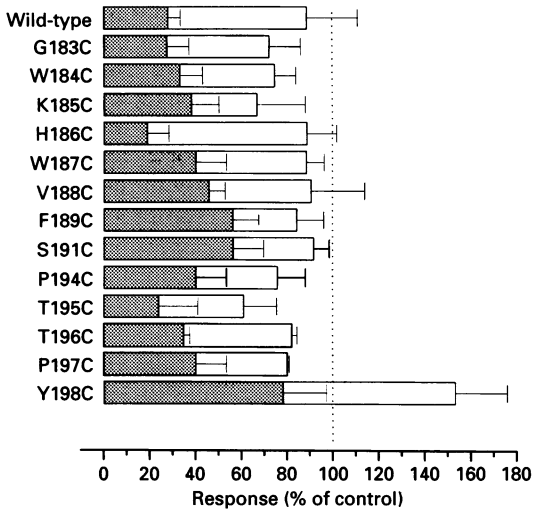

Figure 2 Effect of DTT on wild-type and cysteine-substitution mutations

Oocytes were challenged with a half-maximal concentration of ACh before and after a 1 min exposure to either 1 (□) or 5 (■) mM DTT. Data are plotted as the percentage of the control response and are means ± S.D. The dotted line indicates the control value before DTT treatment. Only the response of αY198C after 1 mM DTT was significantly different from that of the wild type (Student's *t* test; $P < 0.00234$).

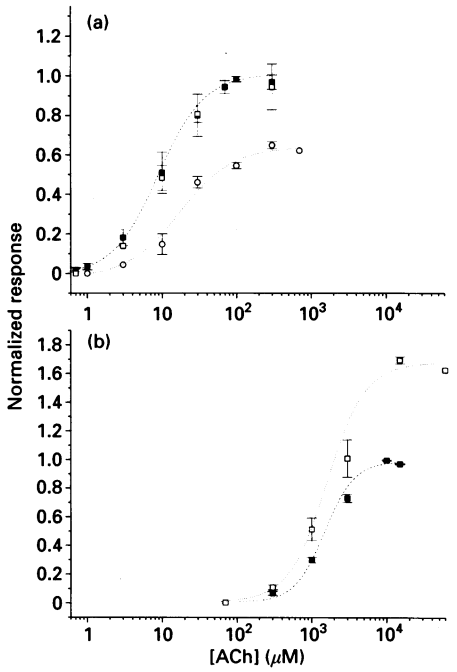

Figure 3 Dose–response profiles of the wild-type (a) and αY198C mutation (b) before and after exposure to DTT

Plotted data are from oocytes for which a complete dose–response profile was obtained both before and after exposure to DTT. Broken lines are derived from a logistic fit of each data set. (**a**) Wild-type: ■, control; □, 1 mM DTT; ○, 5 mM DTT. Wild-type control: EC_{50} 10 ± 1.5 μM, Hill coefficient 1.4; wild-type 5 mM DTT: EC_{50} 17 ± 3 μM, Hill coefficient 1.3. (**b**) αY198C: ■, control; □, 1 mM DTT. αY198C control: EC_{50} 1.5 ± 0.17 mM, Hill coefficient 2.1; αY198C 1 mM DTT: EC_{50} 1.6 ± 0.2 mM, Hill coefficient 1.9.

remaining mutant α-subunits appear to assemble with β-, γ- and δ-subunits to form functional AChRs at levels comparable with that of the wild-type α-subunit.

One of our major concerns was the possible disruption of the structure of the binding site as the result of formation of disulphide bonds between introduced cysteines and the native cysteines at positions 192 and 193. This was of particular concern in those mutants in which the apparent agonist affinity was markedly different from the wild type. To examine this we looked at the effects of the reducing agent DTT on currents elicited by a half-maximal concentration of ACh for both wild-type and mutant AChRs, as shown in Figure 2. It is well established that DTT reduction of the disulphide between αC192 and αC193 can inhibit the *Torpedo* AChR [27]; we find that inhibition of the wild-type AChR expressed in oocytes is seen after brief exposure to 5 mM DTT, but not to 1 mM DTT. With the exception of the αY198C mutant, the sensitivity of all cysteine mutants to DTT resembles that of the wild-type receptor: little or no inhibition at 1 mM DTT, and substantial inhibition at 5 mM DTT.

In contrast with the other cysteine mutants, a brief exposure of the αY198C mutant to 1 mM DTT produces a significant increase in the ACh-evoked current (Figure 2). Exposure of the αY198C mutant to 5 mM DTT, however, gives rise to a substantial decrease in ACh responsiveness similar to that seen with the wild-type. We examined the nature of the DTT effect on this mutant further by comparing dose–response profiles of wild-type and αY198C receptors before and after exposure to 1 mM DTT, as shown in Figure 3. The effect of 1 mM DTT on the αY198C is largely one of an increase in the peak current, with very little effect on the EC_{50} for activation. Lower concentrations of reducing agent (< 1 mM) had negligible effects on both the wild type and the other cysteine-substitution mutants, suggesting that the biphasic nature of the DTT effect was unique to the αY198C mutation.

We next wanted to test whether any of the cysteine-substitution mutations were sensitive to modification by a thiol-specific

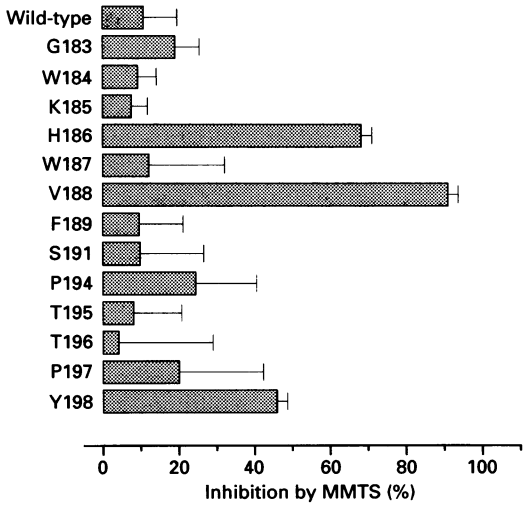

Figure 4 Effect of MMTS on wild-type and cysteine-substitution mutations

Oocytes were challenged with a half-maximal concentration of ACh before and after a 90 s exposure to 5 mM MMTS. Data are plotted as percentage inhibition of control and are means ± S.D. Inhibition seen in three mutants, αH186C, αV188C and αY198C, was significantly different from that of the wild type (Student's *t* test; $P < 0.001$).

reagent, MMTS. This compound specifically reacts with free thiol groups converting them into thio–methyl adducts. We measured responses of both wild-type and mutant receptors to a

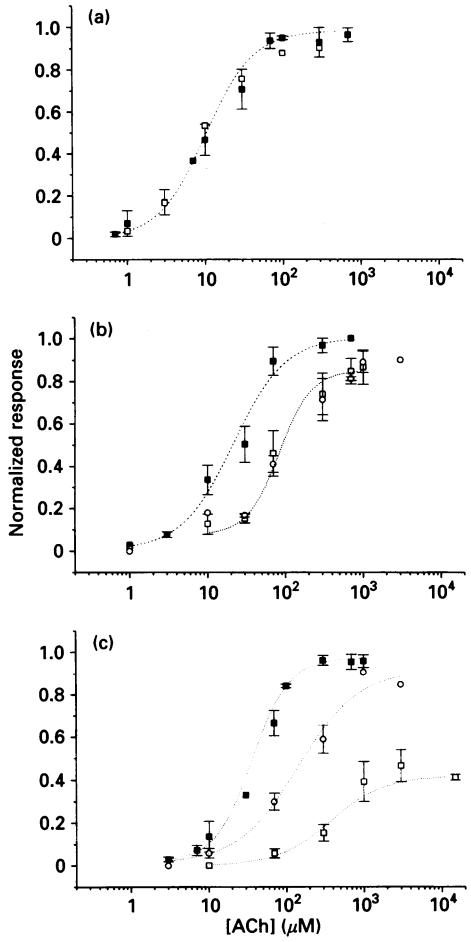

Figure 5 Dose–response profiles of wild type (a), αH186C (b) and αV188C (c) before and after exposure to MMTS

Plotted data are from oocytes for which a complete dose–response curve was obtained both before and after exposure to MMTS. Broken lines are derived from a logistic fit of each data set. (**a**) Wild type: ■, control; □, 5 mM MMTS; wild-type EC_{50} 10±1.5 μM, Hill coefficient 1.4. (**b**) αH186C: ■, control; □, 5 mM MMTS; ○, 1 mM DTT after 5 mM MMTS; αH186C control: EC_{50} 22±1.9 μM, Hill coefficient 1.3; αH186C after MMTS: EC_{50} 84±19 μM, Hill coefficient 1.9. (**c**) αV188C: ■, control; □, 5 mM MMTS; ○, 1 mM DTT after 5 mM MMTS; αV188C control: EC_{50} 37±5.6 μM, Hill coefficient 1.8; αV188C after MMTS: EC_{50} 370±120 μM, Hill coefficient 1.2; αV188C after DTT and MMTS: EC_{50} 140±33 μM, Hill coefficient 1.1.

half-maximal concentration of ACh before and after a 90 s exposure to 5 mM MMTS. The results are shown in Figure 4. MMTS exposure has little or no effect on the ACh responses of wild-type AChRs and most of the cysteine-substitution mutations. We cannot at present distinguish between two explanations for the absence of an effect in these cases: either the modification does not occur, or it occurs but has no effect on receptor function.

The ACh responses of three mutants, αH186C, αV188C and αY198C, are significantly inhibited after exposure to MMTS. Two of these mutants, αH186C and αV188C, were analysed further, as shown in Figure 5. We looked at changes in both the EC_{50} and the maximal response for ACh after MMTS treatment. We also examined the extent to which these effects were reversible by low concentrations of DTT, since the mixed disulphide formed by reaction between MMTS and the cysteine should be readily reducible.

AChRs with the αH186C mutation show a rightward shift in the dose–response curve, but little effect on the peak response elicited by ACh (Figure 5b). The effect of modification is not reversed by DTT. The effects of MMTS on the αV188C mutation are qualitatively different from those seen with the αH186C mutant (Figure 5c). In this case, modified receptors display a rightward shift in EC_{50}, and a decrease in the maximal response to ACh; both of these effects are at least partially reversed by exposure to 1 mM DTT.

DISCUSSION

Structural analysis of AChRs, as with other multimeric integral membrane proteins, is severely limited by an inability to apply X-ray-crystallographic and magnetic-resonance techniques that have been successfully used in studies of smaller soluble proteins. In this report we have described our initial results using cysteine-substitution mutagenesis to investigate AChR structure and function. This method should allow us to apply a number of biochemical and spectroscopic techniques to examine these further. We have focused our initial efforts on the α-subunit domain which is likely to include major structural determinants of the ACh-binding site.

The fact that most of the substitutions do not significantly alter functional properties of the receptor indicates that we have not dramatically altered the three-dimensional structure of the binding site. This was a particular concern because of the presence of a critical disulphide in this region. Two of the 14 mutants are exceptions to this conclusion: αY198C and αY190C. We are not surprised that these two positions were the most sensitive to cysteine substitution; previous site-directed mutation of these conserved tyrosines has demonstrated their importance in agonist activation. The apparent absence of functional AChRs with the αY190C mutation was somewhat surprising, given a previous report of the expression of this mutation in transfected mammalian cells [28].

The αY198C mutant exhibited a distinct and unusual increase in peak response after exposure to DTT, and also displayed an EC_{50} for agonist two orders of magnitude higher than that of the wild type. One possible explanation for this result is that some of the mutant receptors have an aberrant disulphide between C198 and one of the native cysteines at positions 192 or 193. Receptors containing such aberrant disulphides would probably be non-functional until the disulphide is reduced, resulting in an increase in the number of functional receptors and consequently an increase in peak response. Consistent with the idea of an aberrant disulphide is the fact that other mutations at position 198 (such as αY198F) do not show this increase in peak response after exposure to reducing agent (J. T. McLaughlin, unpublished work).

In the segment between α183 and α197, we find that substitution of individual cysteines at 12 of the 15 residues produces only moderate changes in the apparent affinity for ACh. Exposure of AChRs with substituted cysteines to MMTS altered functional responses to agonist for only two of the cysteine mutants: αH186C and αV188C. The absence of an effect at other sites can be explained by one of two possibilities: (1) the inaccessibility of the side chain to reagent or (2) the absence of an effect of the modification on ACh-evoked responses. Distinguishing between these two possibilities will provide information that should be of use in modelling the structure of the agonist-binding domain. The major finding of this study is that agonist activation of two cysteine substitutions, αH186C and αV188C, is subject to inhibition by MMTS modification. In addition, the functional

effects of the MMTS modifications at these sites are qualitatively different.

At its simplest, the process of agonist activation of the AChR can be thought of as a two-step process: binding of ACh and the conformational change between closed and open states of the liganded channel. Observed changes in dose–response relationships can be due to changes in either, or both, of these steps [29,30]. Because the efficacy of ACh for the wild-type channel is high (open/closed ratio for liganded AChR is approx. 14 [31]), chemical modifications (or mutations) which simply shift the dose–response curve can be due to a change in either of the two steps. The effect of MMTS modification at αH186C is such a case; for this mutant, we cannot distinguish an effect on binding from an effect on efficacy. In contrast, the MMTS-treated αV188C mutant exhibited both a shift in EC_{50} and a decline in the peak current. The change in peak current, particularly in the light of the normally high efficacy of ACh, indicates that the modification has altered the ability of the receptor to undergo the closed-to-open transition. Thus, although the two sites (186 and 188) are separated by only a single residue, it is possible that the inhibition resulting from MMTS modification could be due to effects on two different steps in the activation process: inhibition of ACh binding (αH186C) and inhibition of the closed-to-open transition (αV188C).

Modification of the αH186C and αV188C mutations by MMTS can also be distinguished in terms of their relative reversibility by DTT. In the case of the αH186C mutant, we find that the effect of modification is not reversed by 1 mM DTT. This could be due to an 'irreversible' change in receptor conformation or a change in accessibility of the residue after modification, such that the DTT would not have access to the thio–methyl adduct. The effect of modification of the αV188C mutant is, in contrast, substantially reversible by 1 mM DTT. These observations are consistent with a mechanistic distinction between the effects of MMTS on these two cysteine substitutions.

MMTS concentrations as low as 250 μM produce the same effects as higher concentrations on both αH186C and αV188C, suggesting that the extent of modification we observe is complete and not limited by MMTS concentration or by reaction time (results not shown). Nevertheless, our results could be explained by a heterogeneous population of partially, completely and unmodified receptors that have distinct dose–response profiles. For example, modification of the αV188C mutant at only one of the two α-subunits could cause a shift in the dose–response curve to the right, whereas complete modification could abolish agonist-dependent activation (reducing the peak response). The observed dose–response curve would represent a summation of responses of unmodified and partially modified receptors. Similarly, we cannot rule out a change in single-channel properties (such as mean open time or single-channel conductance) as an explanation for the effect of DTT on αY198C. Single-channel analysis of the cysteine mutations, both before and after exposure to modifying reagents, should help to clarify the underlying changes responsible for our observations. Site-directed mutagenesis has become an important tool in the identification of residues critical to ion channel structure and function; despite this, interpretation of results from such experiments is not always straightforward. One of the most significant advantages of the cysteine-substitution technique is that it allows for examination of the same population of channels before and after modification. In this sense, chemical modification of engineered cysteines may be thought of as *in situ* or real-time mutagenesis. This, together with the many biochemical tools available for characterization of cysteines, promises to make cysteine-substitution mutagenesis an important approach to elucidating the structure and function of ligand-gated channels such as the AChR.

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